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ORIGINAL PAPER

Structural analysis of Cu(II) ligation to the 5′-GMP nucleotide by pulse EPR spectroscopy

Maria Grazia Santangelo · Alfredo Medina-Molner · Arthur Schweiger · George Mitrikas · Bernhard Spingler

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Keywords Copper · Electron–nuclear double resonance · Hyperfine sublevel correlation spectroscopy · Pulse electron paramagnetic resonance spectroscopy · Nucleotide

Abbreviations

CW	Continuous wave
DMSO	Dimethyl sulfoxide
ENDOR	Electron-nuclear double resonance
EPR	Electron paramagnetic resonance
5′-GMP	Guanosine 5′-monophosphate
HYSCORE	Hyperfine sublevel correlation
Triflate	Trifluoromethanesulfonate

Arthur Schweiger died on 4 January 2006.

M. G. Santangelo · A. Schweiger · G. Mitrikas
Laboratory of Physical Chemistry,
ETH Zurich,
8093 Zurich, Switzerland

A. Medina-Molner · B. Spingler (✉)
Institute of Inorganic Chemistry,
University of Zurich,
8057 Zurich, Switzerland
e-mail: spingler@aci.unizh.ch

G. Mitrikas (✉)
Institute of Materials Science,
NCSR ''Demokritos'', Aghia Paraskevi Attikis,
15310 Athens, Greece
e-mail: george.mitrikas@phys.chem.ethz.ch

Introduction

The interaction of metal complexes with oligonucleotides is one of the central topics in bioinorganic chemistry. We have been interested in metal complexes that are able to induce Z-DNA [1]. Copper complexes of substituted 1,4,7,10,13-pentaazacyclohexadecane-14,16-dione are able to convert the right-handed B form of poly d(GC) to the corresponding left-handed Z form [2]. Preformed Z-DNA crystals that were soaked with copper salts bound the metal ions exclusively via N7 of guanine [3]. On the other hand, simple copper salts are known to

denature the same DNA sequence [4]. Sundaralingam and Carrabine [5] proposed a model to explain this behavior. We started a research program in order to understand why copper(II) as an aquated ion denatures DNA on the one hand and induces the conformational change to Z-DNA in the form of an azamacrocyclic complex on the other hand. Copper is an ideal metal for electron paramagnetic resonance (EPR) spectroscopy [6–8]. In order to successfully study the interaction between copper and oligonucleotides, one has first to study the copper–mononucleotide system. We were rather surprised that this has not yet been done with the help of pulse EPR [9–11]. These techniques allow, together with the use of isotope-labeled nucleotides, the determination of the distance between the paramagnetic copper center and selected atoms around it within a radius of not more than 6 Å. In this paper, based on pulse EPR experiments, we are able to describe the geometry of copper(II) bound to guanosine 5′-monophosphate (5′-GMP) in solution. This study is the entry point to further characterize copper–oligonucleotide systems.

Materials and methods

Sample preparation

We prepared four different samples: sample 1, copper(II) trifluoromethanesulfonate (triflate) in 50% dimethyl sulfoxide (DMSO) and 50% H_2O; sample 2, copper(II) triflate and 5′-GMP (Fig. 1a) in 50% DMSO and 50% H_2O; sample 3, copper(II) triflate and 5′-GMP with deuterium atoms instead of the position 8 (D8-5′-GMP, Fig. 1b) in 50% DMSO and 50% H_2O; sample 4, copper(II) triflate and 5′-GMP deuterated at all the exchangeable protons (H8-d_5-5′-GMP, Fig. 1c) in 50% DMSO-d_6 and 50% D_2O. All reagents and nucleotides were purchased from Fluka at the highest purity available. In all samples the metal concentration was 2 mM. For the 5′-GMP-containing samples (samples 2, 3, and 4) the metal-to-ligand ratio was 1:1. D8-5′-GMP was prepared by overnight treatment of 5′-GMP at 60 °C with 4 equiv of triethylamine in D_2O. Excess reagent was removed by repeated lyophilization to dryness from H_2O [12]. ^{1}H NMR showed the absence of any triethylamine. The H8-d_5-5′-GMP ligand was prepared by overnight treatment in D_2O. Excess reagent was removed by lyophilization. The pH of all the samples was determined to be 6.4.

Spectroscopy

Continuous wave (CW) EPR measurements at X-band were carried out using a Bruker E500 spectrometer equipped with a Bruker super-high-Q cavity. Experimental conditions were as follows: microwave frequency, 9.5 GHz; microwave power incident to the cavity, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT. Cooling of the sample was performed with a liquid-nitrogen finger Dewar vessel to 130 K.

The X-band pulse EPR experiments were performed with a Bruker E580 spectrometer (microwave frequency, 9.72 GHz) equipped with a liquid-helium cryostat from Oxford Instruments. All experiments were done at 20 K and a repetition rate of 1 kHz. The magnetic field was measured with a Bruker ER083 CS Gauss meter.

Hyperfine sublevel correlation (HYSCORE) spectroscopy [13, 14], with the pulse sequence $\pi/2$–τ–$\pi/2$–t_1–π–t_2–$\pi/2$–τ–echo, is a two-dimensional experiment that correlates nuclear frequencies in one electron spin manifold to nuclear frequencies in the other electron spin manifold. The HYSCORE spectra were recorded with the following instrumental parameters: $t_{\pi/2} = t_\pi = 16$ ns; starting values of the two variable times t_1 and t_2, 56 ns; time increment, $\Delta t = 16$ ns (data matrix 350 × 350). To avoid blind spots, spectra with different τ values were recorded and added. An eight-step phase cycle was used to remove unwanted echoes.

The Davies electron-nuclear double resonance (ENDOR) [13, 15] experiments were carried out with the pulse sequence π–T–$\pi/2$–τ–π–τ–echo, with microwave pulse lengths of 32, 16, and 32 ns, respectively, and an interpulse time τ of 400 ns. A radiofrequency π pulse of variable frequency and a length of 9 μs was applied during time T (10 μs).

The Mims ENDOR [13, 16] experiments were performed using the pulse sequence $\pi/2$–τ–$\pi/2$–T–$\pi/2$–τ–echo, with microwave pulse lengths of 16 ns and an interpulse time of $\tau = 500$ ns. During time T (10 μs) a radiofrequency

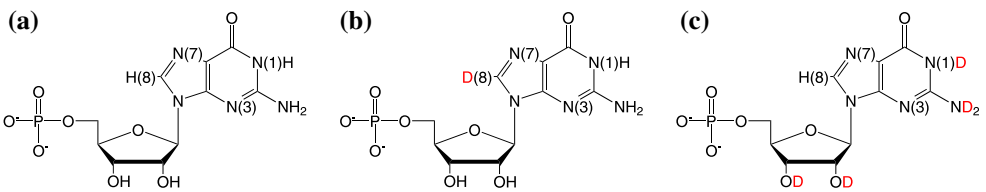

Fig. 1 Chemical structures of guanosine 5′-monophosphate (5′-GMP) and selectively deuterated 5′-GMP: **a** 5′-GMP; **b** D8-5′-GMP; **c** H8-d_5-5′-GMP

pulse with variable frequency and a length of 9 μs was applied.

Both ENDOR and HYSCORE experiments were carried out at different observer positions that correspond to different orientations of the molecules with respect to $\mathbf{B}_0$ (orientation selectivity) [13].

Data manipulation

The data were processed with the program MATLAB 7.2 (MathWorks, Natick, MA, USA). The time traces of the HYSCORE spectra were baseline-corrected with a two-order exponential, apodized with a Gaussian window and zero-filled. After a two-dimensional Fourier transformation the absolute-value spectra were calculated. The HYSCORE spectra recorded with different τ values were added to eliminate τ-dependent blind spots.

The CW EPR and ENDOR spectra were simulated with the EasySpin package [17]. For the CW EPR simulations the hyperfine coupling shift due to the two copper isotopes ^{63}Cu and ^{65}Cu was taken into account. The HYSCORE spectra were simulated with a program written in-house [18].

Theory

The spin Hamiltonian for a spin system with a Cu(II) ion (electronic configuration $3d^9$, $S = 1/2$) and m nuclei with spins I is given by Eq. 1 [13]:

$$H = \beta_e \mathbf{B}_0 \mathbf{g} \mathbf{S}/h + \sum_k^m \mathbf{S} \mathbf{A}_k \mathbf{I}_k - \beta_n \sum_k^m g_{n,k} \mathbf{B}_0 \mathbf{I}_k/h + \sum_{I_k > 1/2} \mathbf{I}_k \mathbf{Q}_k \mathbf{I}_k, \quad (1)$$

where the terms describe the electron Zeeman interaction, the hyperfine interaction, the nuclear Zeeman interaction, and the nuclear quadrupole interaction (for nuclei with $I > 1/2$). The electron Zeeman interaction is characterized by the **g** tensor that is essentially determined by the metal ion and the directly coordinated ligand atoms. The g values observed in the EPR spectrum together with the metal hyperfine coupling can often be used as a fingerprint to identify the metal ion and to provide information on the symmetry of the paramagnetic center. The ligand hyperfine interaction can be written as the sum of the isotropic interaction or Fermi contact interaction $H_F = a_{iso}\mathbf{SI}$ and the electron–nuclear dipole–dipole coupling $H_{DD} = \mathbf{STI}$. Here a_{iso} is the isotropic hyperfine coupling constant which is directly related to $|\psi_0(0)|^2$, the electron spin density at the nucleus and matrix **T** describes the anisotropic dipole–dipole coupling. For protons, the anisotropic part of the hyperfine interaction can be approximated using the point-dipole model assuming that the distance r between the unpaired electron and the proton is larger than 0.25 nm. In this case, the point-dipole formula (Eq. 2)

$$\mathbf{T} = \frac{\mu_0}{4\pi h} g_e \beta_e g_n \beta_n \frac{(3\tilde{\mathbf{n}}\mathbf{n} - \mathbf{1})}{r^3}, \quad (2)$$

where **n** is the electron–nucleus unit vector, can be used to calculate the electron–nuclear distance and orientation with respect to the **g** tensor.

For nuclear spins larger than 1/2 , such those of as ^{14}N and ^{2}H with $I = 1$, the nuclear quadrupole principal values $[Q_x, Q_y, Q_z] = [-K(1 - \eta), -K(1 + \eta), 2K]$ of the traceless **Q** matrix are usually expressed by the quadrupole coupling constant $K = e^2qQ/4I(2I - 1)h$ and the asymmetry parameter $\eta = (Q_x - Q_y)/Q_z$, where Q is the nuclear quadrupole moment and eq is the electric field gradient.

For an $S = 1/2$, $I = 1/2$ spin system there are two single-quantum nuclear transitions ($|\Delta m_S| = 0$, $|\Delta m_I| = 1$). For the special case when **g**, **A**, and **Q** are coaxial and $\mathbf{B}_0$ is along one of the principal axes, the first-order frequencies are given by Eq. 3:

$$\nu_i = |\nu_I \pm A_i/2|, \quad (3)$$

where ν_I is the nuclear Zeeman frequency and A_i ($i = x, y, z$) is one of the principal hyperfine values. For $\nu_I > |A_i/2|$ (weak coupling case) the two frequencies are centered at ν_I and separated by A_i. For $\nu_I < |A_i/2|$ (strong coupling case) the two frequencies are centered at $A_i/2$ and split by $2\nu_I$.

For an $S = 1/2$, $I = 1$ spin system there are four single-quantum nuclear frequencies given by Eq. 4,

$$\nu_i = |\nu_I \pm A_i/2 \pm 3/2\ Q_i|, \quad (4)$$

and two double-quantum nuclear transitions ($|\Delta m_S| = 0$, $|\Delta m_I| = 2$). If the anisotropic hyperfine coupling is small compared to the isotropic hyperfine interaction and the nuclear quadrupole interaction, the latter frequencies are given by Eq. 5 [19]:

$$\nu_{\alpha,\beta}^{dq} = 2\left[(a_{iso}/2 \pm \nu_I)^2 + K^2(3 + \eta^2)\right]^{1/2}. \quad (5)$$

ENDOR and HYSCORE experiments are especially suited for accurately measuring nuclear transition frequencies which are related to the hyperfine and nuclear quadrupole interactions of the spin system. ENDOR spectra consist of peaks that correspond mainly to single-quantum transition frequencies, whereas the HYSCORE spectrum of an $S = 1/2$, $I = 1$ system is usually dominated by cross-peaks between the double-quantum frequencies.

Results and discussion

The pK_a value of the second deprotonation step of the phosphate group in 5′-GMP was determined to be 6.25 ± 0.02 [20]. Addition of 1 equiv of copper lowers this pK_a value down to 4.9 ± 0.3 [21]. The pH of all the samples measured was 6.4 ± 0.1; therefore, all the Cu(II)–5′-GMP complexes formed had a neutral charge.

CW EPR spectra

The frozen-solution X-band EPR spectra of the copper(II) triflate sample (sample 1) and the Cu(II)–5′-GMP samples (samples 2, 3, and 4) together with their simulations are shown in Fig. 2. All spectra revealed features that are typical for Cu(II) complexes with a $(d_{x^2-y^2})^1$ ground state ($g_\| > g_\perp$) [22] Table 1). The comparison of the g values and the Cu(II) hyperfine parameters between copper(II) triflate (sample 1) and Cu(II)–5′-GMP (samples 2, 3, and 4) indicates a metal–guanosine interaction. Indeed the EPR parameters of copper(II) triflate are typical for Cu(II) complexes with four oxygen atoms as equatorial ligands [23]. Instead the g values and Cu hyperfine parameters of Cu(II)–5′-GMP complexes are typical for Cu(II) complexes with either three oxygen atoms and one nitrogen atom as equatorial ligands or four oxygen as equatorial ligands. We cannot distinguish between the two possibilities by the inspection of CW EPR spectra. However, application of pulse EPR spectroscopy (see ‘‘^{14}N Davies ENDOR spectra’’) ruled out the second possibility, confirming that the Cu ion is coordinated to three oxygen atoms and one nitrogen atom. This is an indication that the surrounding environment of the Cu(II) ion changed when the 5′-GMP ligand was added. Moreover the g values and the hyperfine parameters of samples 2, 3, and 4 are very similar to each other. This is an indication that the interaction between the Cu(II) ion and the guanosine nucleotide is the same for all three slightly different kinds of 5′-GMP ligands that we used. Although the CW EPR data provide evidence of a coordination of the copper metal by the 5′-GMP nucleotide, detailed information about the metal ion site cannot be obtained. Further insight into the metal ion site is provided by pulse EPR techniques.

^{14}N Davies ENDOR spectra

The Davies ENDOR spectra of Cu(II)–H8-d_5-5′-GMP (sample 4) at different magnetic field positions together with their simulations are shown in Fig. 3. Davies ENDOR experiments are very well suited for measuring strong hyperfine couplings. The spectra are characterized by two broad peaks centered at approximately $|a/2| = 16$ MHz and split by twice the nitrogen nuclear Zeeman frequency, $2\nu_N$. This is typical for a *strongly* coupled nitrogen nucleus with $|a/2| > |\nu_N|$ [13]. This result combined with the CW EPR parameters validates the hypothesis that we have a copper complex with three oxygen atoms and one nitrogen atom as equatorial ligands. The additional splitting observed in all spectra is assigned to the nuclear quadrupole interaction. Moreover, signals from weakly coupled protons, which overlap with signals from the strongly coupled nitrogen, were suppressed by using short microwave pulses. This effect is known as hyperfine contrast-selective ENDOR [13]. Davies ENDOR measurements at different observer positions (thin traces) and their simulations (thick traces) allow for the estimation of the principal values of the hyperfine, **A**, and nuclear quadrupole, **Q**, coupling tensors: $(A_x, A_y, A_z) = (32.0, 38.0, 31.0) \pm 0.2$ MHz, and $(Q_x, Q_y, Q_z) = (2.1, -1.5, -0.6) \pm 0.5$ MHz. Because the larger hyperfine coupling occurs at the observer position corresponding to $g_\perp$, we conclude that this strongly coupled nitrogen occupies an equatorial position. Although the N7 atom is the most plausible binding site of the ligand, from these data we are not able to identify, in a definitive way, the identity of this strongly coupled nitrogen. However, with the knowledge of the hyperfine and nuclear quadrupole interactions between the unpaired electron of the Cu(II) ion and the other magnetic nuclei, it is possible to obtain detailed insight into the local environment of the paramagnetic center, and to validate the hypothesis that the Cu(II) ion is directly coordinated to atom N7.

^{1}H HYSCORE spectra

To get information about the weakly coupled nuclei, the HYSCORE technique was utilized. The X-band proton spectra of samples 2, 3, and 4 are shown in Fig. 4 together with the corresponding simulations. The HYSCORE spectrum of the Cu(II)–5′-GMP complex (sample 2, Fig. 4a) shows at least two types of weakly coupled protons. One type is characterized by an intense ridge close to the antidiagonal at the proton Larmor frequency (approximately 14 MHz) and can be assigned either to protons of water molecules coordinated in axial positions or/and to weakly coupled protons of solvent molecules [24]. The other type of proton HYSCORE signal is distinguished by a broad ridge shifted away from the antidiagonal. This spectrum can be simulated with the hyperfine parameters $(A_x, A_y, A_z) = (-6.3, -6.3, 9.3) \pm 0.2$ MHz and Euler angles $[\alpha, \beta, \gamma] = [0, 90, 0]°$, which correspond to an isotropic hyperfine coupling constant of $a_{iso} = -1.1 \pm 0.2$ MHz and a dipolar coupling constant of $T = 5.2 \pm 0.2$ MHz. In the point-dipole approximation, the latter value corresponds to a point-dipole distance of 2.5 ± 0.1 Å. These weakly coupled protons can be assigned either to ligand protons of the nucleotide or to water

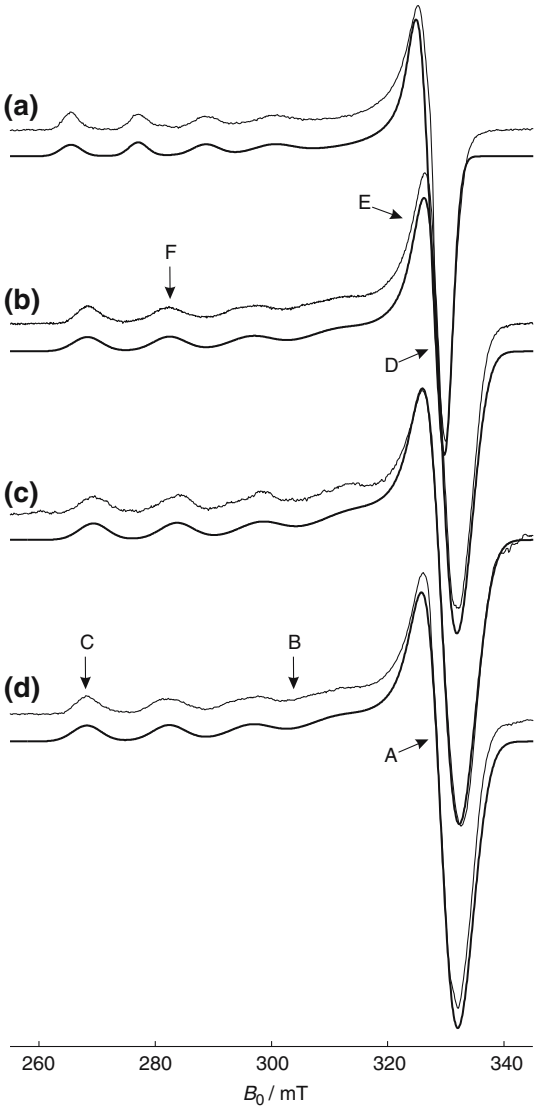

Fig. 2 X-band continuous wave electron paramagnetic resonance spectra taken at 130 K: *a* Copper(II)trifluoromethanesulfonate in dimethyl sulfoxide (DMSO)/H_2O solution (sample 1); *b* Cu(II)–5′-GMP complex in DMSO/H_2O solution (sample 2); *c* Cu(II)–D8-5′-GMP complex in DMSO/H_2O solution (sample 3); *d* Cu(II)-H8-d_5-5′-GMP complex in DMSO-d_6/D_2O solution (sample 4). *Thin traces* experiments, *thick traces* simulations. *A–F* observer positions used for the Davies and Mims electron-nuclear double resonance (ENDOR) measurements

Table 1 *g* values and ^{63}Cu hyperfine parameters

Sample	$g_\perp$ (±0.005)	$g_\parallel$ (±0.005)	$\lvert A_\perp \rvert$ (±10) (MHz)	$\lvert A_\parallel \rvert$ (±10) (MHz)
1	2.079	2.406	25	382
2	2.072	2.358	15	455
3	2.067	2.340	15	460
4	2.069	2.353	15	455

See ‘‘Sample preparation’’ for an explanation of the samples

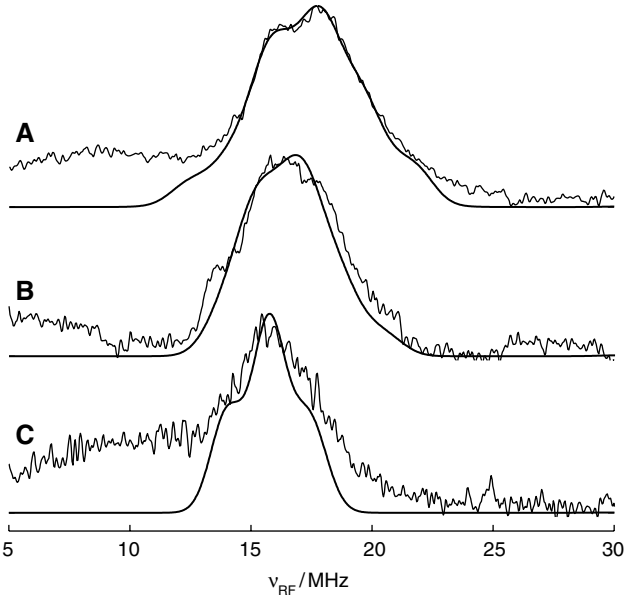

Fig. 3 X-band Davies ENDOR spectra of Cu(II)–H8-d_5-5′-GMP in DMSO-d_6/D_2O solution (sample 4) taken at observer positions *A–C* (see Fig. 2, spectra d). *Thin traces* experiments, *thick traces* simulations

molecules directly coordinated to the copper. In order to be able to distinguish between these options we repeated the same experiment for the Cu(II)–D8-5′-GMP sample (sample 3) with a deuterium nucleus in the position 8 (Fig. 1b). New peaks from a deuterium nucleus appeared at low frequency (for details see ‘‘^{14}N, ^{2}H HYSCORE spectra’’; Fig. 5b). Moreover, the proton HYSCORE spectrum in Fig. 4b can be simulated with the same hyperfine parameters we found for sample 2 (Table 2). This finding indicates that in both complexes we can detect the same type of weakly coupled protons. This proton peak certainly cannot be assigned to H8 because in sample 3 there is a deuterium at this position. Moreover the hyperfine couplings and the distance obtained from our analysis are compatible with the hyperfine couplings and the distance of water protons in $Cu[(H_2O)_6]^{2+}$ complexes determined in a HYSCORE [24] and a single-crystal CW ENDOR study [25]. These considerations confirm the hypothesis that the aforementioned HYSCORE signal can be assigned to protons of water molecules directly coordinated to the copper center. To study the hyperfine interaction between the unpaired electron of the copper ion and the H8 proton of the nucleotide, we measured the HYSCORE spectrum of the Cu(II)–H8-d_5-5′-GMP sample (sample 4) in 50% DMSO-d_6 and 50% D_2O. In this sample, all the exchangeable protons have been replaced with deuterium nuclei (Fig. 1c). The experimental spectrum in Fig. 4c consists of proton peaks close to the antidiagonal at the proton Larmor frequency and combination peaks

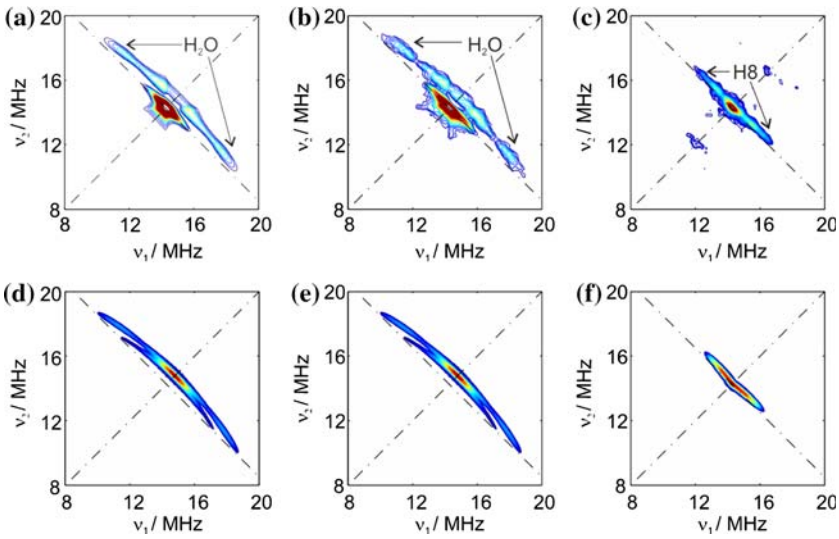

Fig. 4 X-band proton hyperfine sublevel correlation (HYSCORE) spectra measured at a magnetic field position close to $g_{\perp}$. **a** Cu(II)–5′-GMP (sample 2), τ = 100, 120, 140, and 160 ns; **b** Cu(II)–D8-5′-GMP (sample 3), τ = 100, 120, 140, and 160 ns; **c** Cu(II)–H8-d_5-5′-GMP (sample 4); τ = 100, 120, 140, 160, 400, 450, and 500 ns; **d–f** corresponding simulations. The *antidiagonal lines* are given for ν_{1H}

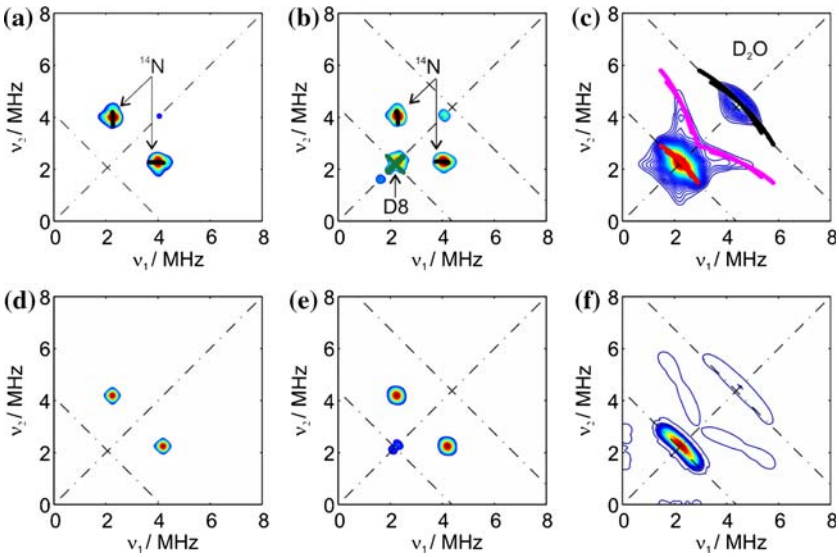

Fig. 5 X-band nitrogen and deuterium HYSCORE spectra measured at a magnetic field position close to $g_{\perp}$. **a** Cu(II)–5′-GMP (sample 2), τ = 100, 120, 140, and 160 ns; **b** Cu(II)–D8-5′-GMP (sample 3), τ = 100, 120, 140, and 160 ns; **c** Cu(II)–H8-d_5-5′-GMP (sample 4); τ = 100, 120, 140, 160, 400, 450, and 500 ns; **d–f** corresponding simulations. The *antidiagonal lines* are given for ν_{2H} and $2\nu_{2H}$

Table 2 ^{1}H hyperfine parameters, Euler angles, and derived distances

Sample	$A_{\perp}$ (±0.2) (MHz)	$A_{\parallel}$ (±0.2) (MHz)	[α, β, γ] (°)	a_{iso} (±0.2) (MHz)	T (±0.2) (MHz)	r (±0.1) (Å)
2	–6.3	9.3	[0, 90, 0]	–1.1	5.2	2.5
3	–6.3	9.3	[0, 90, 0]	–1.1	5.2	2.5
4	–0.3	7.5	[0, 30, 0]	2.3	2.6	3.1

between the proton and the deuterium frequency. The proton signal can be simulated with the hyperfine parameters (A_x, A_y, A_z) = (–0.3, –0.3, 7.5) ± 0.2 MHz and Euler angles [α, β, γ] = [0, 30, 0]°, which correspond to an isotropic hyperfine coupling constant of a_{iso} = 2.3 ± 0.2 MHz and a dipolar coupling constant of T = 2.6 ± 0.2 MHz. In the point-dipole approximation, the latter value corresponds to a point-dipole distance of 3.1 ± 0.1 Å. This weakly coupled proton can be exclusively assigned to H8 since in this sample there are no other protons in such close proximity to the copper center. Moreover, our data are in agreement with the imidazole proton measured in a copper–histidine complex by W-band Davies ENDOR [26]. The distance between the H8 proton and the paramagnetic center is compatible with the hypothesis that the Cu(II) ion is directly coordinated to nitrogen N7.

^{14}N, ^{2}H HYSCORE spectra

The low-frequency regions of the HYSCORE spectra of samples 2, 3, and 4 are shown in Fig. 5 together with the corresponding simulations. The HYSCORE spectrum of Cu(II)–5′-GMP (sample 2) (Fig. 5a) is dominated by cross-peaks that are assigned to double-quantum correlation peaks from ^{14}N and their strong intensity is typical for disordered $S = 1/2$, $I = 1$ spin systems with negligible anisotropic hyperfine coupling [27]. Numerical simulations of the set of spectra recorded at different observer positions (not shown here) yield the following hyperfine and nuclear quadrupole parameters: $(A_x, A_y, A_z) = (1.51, 1.51, 1.63) \pm 0.05$ MHz with Euler angles $[\alpha, \beta, \gamma] = [0, 10, 0]°$ and $|e^2qQ/h| = 2.44 \pm 0.05$ MHz with Euler angles $[\alpha, \beta, \gamma] = [90, 90, 0]°$ and $\eta = 0.56 \pm 0.05$. This nitrogen is characterized by a fairly isotropic hyperfine interaction and a significant quadrupole interaction. The latter can be used as a probe in order to identify this nitrogen. Its comparison with nuclear quadrupole parameters of guanine nitrogens obtained by nuclear quadrupole resonance studies [28] indicates that N1 ($e^2qQ/h = 2.63$ MHz, $\eta = 0.60$) is a more plausible candidate than N7 ($e^2qQ/h = 3.27$ MHz, $\eta = 0.16$). Although other 5′-GMP nitrogens, like N9 ($e^2qQ/h = 1.91$ MHz, $\eta = 0.75$) or N3 (data not available), cannot be excluded, our nuclear quadrupole parameters together with the weak hyperfine coupling strongly suggest that the signal can be assigned to a remote nitrogen (other than N7) of the 5′-GMP ligand. This finding is in line with the hypothesis that the Cu(II) ion is directly coordinated to nitrogen N7, as was suggested by the previous analysis of the proton HYSCORE spectra.

The HYSCORE spectrum of the Cu(II)–D8-5′-GMP sample (sample 3) (Fig. 5b) also shows a similar remote nitrogen pattern. This weakly coupled nitrogen is characterized by the same hyperfine and nuclear quadrupole parameters of the nitrogen as in sample 2. Moreover, in sample 3, a new cross-peak appears close to the ^{2}H Larmor frequency at approximately (2.3, 2.3) MHz, which can be assigned to the deuterium nucleus D8. Since the gyromagnetic ratio for deuterium is approximately 6.5 times smaller than for a proton, the hyperfine interaction is expected to scale as $A(^2\text{H}) = A(^1\text{H})/6.5$. By scaling the hyperfine parameters of the H8 proton observed in sample 4, $(A_x, A_y, A_z) = (-0.05, -0.05, 1.15)$ MHz and Euler angles $[\alpha, \beta, \gamma] = [0, 30, 0]°$, and assuming a small quadrupole interaction with $|e^2qQ/h| = 0.4$ MHz and $\eta = 0.03$ (which is typical for deuterium nuclei bonded to carbon), we could accurately simulate the peak assigned to the deuterium nucleus (Fig. 5b, e). This cross-check further supports our previous assignment of the proton H8.

In Fig. 5c, the HYSCORE spectrum of the Cu(II)–H8-d_5-5′-GMP sample (sample 4) is shown. In this sample we have abundant deuterium nuclei from the ligand and the solvent. This together with suppression effects often encountered in electron spin echo envelope modulation spectroscopy [29] could account for the absence of the remote nitrogen peaks observed in the other two samples. The spectrum is characterized by single-quantum, double-quantum, and combination peaks of deuterium nuclei. It can be simulated by scaling the hyperfine parameters of the protons detected in samples 2 and 3 (ascribed to water molecules directly coordinated to copper), $(A_x, A_y, A_z) = (-0.97, -0.97, 1.43)$ MHz with Euler angles $[\alpha, \beta, \gamma] = [0, 90, 0]°$, and assuming a negligible quadrupole interaction. This result is in agreement with the assignment of the proton HYSCORE spectra of samples 2 and 3 discussed in the previous section.

^{31}P Mims ENDOR spectra

To detect the interaction between the copper center and the ^{31}P nucleus of the phosphate group, the Mims ENDOR technique was used. This method is very useful to detect weak hyperfine couplings from nuclei that are at relatively long distances from the paramagnetic center (typically $r \geq 5$ Å) [30]. A weak ^{31}P coupling should produce a pair of transitions positioned symmetrically about the Larmor frequency for that nucleus. Mims ENDOR spectra of the Cu(II)–5′-GMP sample (sample 2) recorded at different magnetic field positions and their simulations are shown in Fig. 6. The hyperfine values and Euler angles used for the simulation are $(A_x, A_y, A_z) = (-0.20, -0.20, 0.45) \pm 0.05$ MHz and $[\alpha, \beta, \gamma] = [0, 40, 0]°$. For this interaction, essentially no electron delocalization into the ^{31}P nucleus is seen ($A_{iso} = 0.02 \pm 0.02$ MHz) and a dipolar coupling of $T = 0.22 \pm 0.02$ MHz is obtained. This dipolar coupling corresponds to a point-dipole distance $r = 5.3 \pm 0.2$ Å. The X-ray structure analysis of Cu(II) with 5′-GMP not only contains three Cu–5′-GMP molecules in the asymmetric unit but also three different types of interactions between the copper ion and the phosphate group [31]. The copper ion binds by direct coordination to the phosphate or via a two hydrogen bridges mediated via two *cis*-coordinated water molecules, or finally via a single hydrogen bridge to the phosphate mediated by one water molecule that is copper-coordinated. These three different interactions have the following increasing copper to phosphate distance ranges: 3.14–3.27, 4.63, and 5.10–5.52 Å. The distance of 5.3 ± 0.2 Å that we found by the Mims ENDOR method is clearly compatible with the latter type of metal–phosphorus interaction distances observed for water-mediated phosphate ligation. Therefore, we can conclude that the Cu(II) ion is indirectly bound via a single water molecule to a phosphate group.

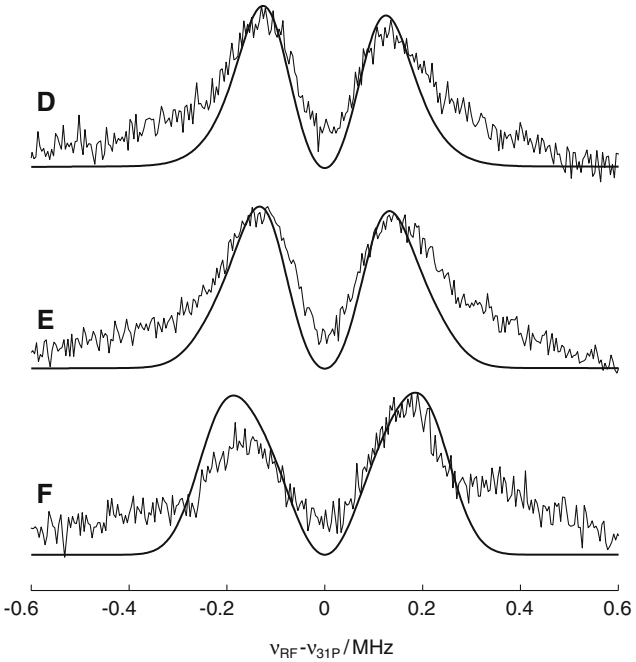

Fig. 6 X-band Mims ENDOR spectra of Cu(II)–5′-GMP (sample 2) taken at observer positions *D–F* (see Fig. 2, spectra b). *Thin traces* experiments, *thick traces* simulations

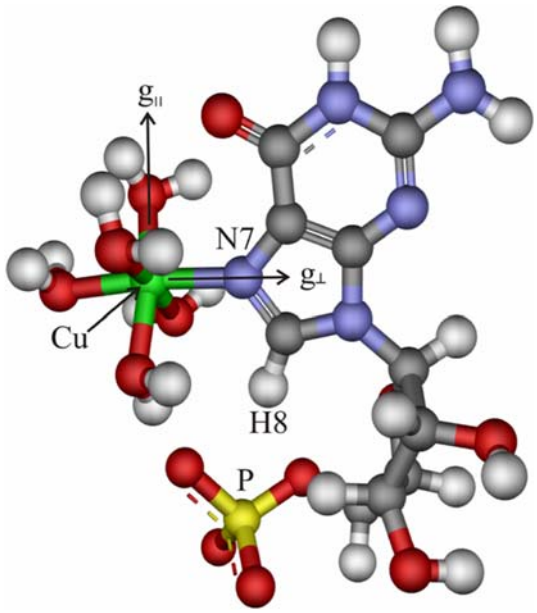

Fig. 7 Molecular model of the $Cu(H_2O)_5$–5′-GMP complex consistent with ENDOR and HYSCORE data. The copper ion is shown in *green* and the phosphorus ion in *yellow*. The figure was created with the help of the program Hyperchem 7.5.1 (Hypercube, Gainesville, FL, USA)

Structural considerations

The *g* values obtained for all the samples indicate that the copper ion in all the samples studied in this work has a $(d_{x^2-y^2})^1$ ground state with a square-planar symmetry. From Davies ENDOR spectra is not possible to determine the number of nitrogens directly coordinate to the copper center. However, from the CW spectrum (Fig. 2, spectra d), we can exclude that more than one nitrogen is directly coordinated to the copper because, since in our samples the metal-to-ligand ratio was 1:1, we do not have evidence of the presence of two species that instead would have formed if more nitrogen atoms were directly coordinated to the copper center. Combining the results of the Davies ENDOR and CW analyses, we can conclude that the copper ion is directly coordinated to only one nitrogen of the nucleotide. Since this latter nitrogen is characterized by a very large isotropic and a very small anisotropic hyperfine constant, we can say that the nitrogen is equatorially coordinated to the copper ion. Moreover we can exclude the presence of a dimer because the CW spectra of all the samples did not show any signals at half the magnetic field (data not shown). A search in the Cambridge Structure Database [32] was undertaken in order to determine to which nitrogen atom(s) of any 9-substituted guanine copper was found to bind. Actually all published X-ray structures of copper binding to a nitrogen of 9-substituted guanine show that the copper is exclusively binding to N7 (e.g., [31, 33]). From the proton HYSCORE spectra we have evidence of a direct coordination of the copper ion by water molecules and in addition by N7 (because of the H8 signal). The molecular model of the Cu(II)–5′-GMP complex consistent with all our data is shown in Fig. 7.

Conclusions

Metal ion coordination to nucleic acids is essential for the biological function of nucleic acids. In this paper we presented a CW and a pulse EPR study of the mononucleotide model system Cu(II)–5′-GMP. We obtained a complete characterization of the structural features of the metal ion bound to the nucleotide. The copper is directly coordinated to N7 of the guanine and to five solvent molecules; one of them forms a hydrogen bridge to the phosphate group. This paper provides a basis for the future characterization of copper–oligonucleotide complexes.

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